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(54) Title:		TREATMENT FOR JUVENILE DIABETES
(57) Abstract		A method for treating diabetes mellitus by administering composition providing a gastrin/CCK receptor ligand, e.g. a gastrin, and an EGF receptor ligand, e.g. TGF α , in an amount sufficient to effect differentiation of pancreatic islet precursor cells to mature insulin-secreting cells. The composition can be administered systemically or expressed <i>in situ</i> by cells transgenically supplemented with one or both of a gastrin/CCK receptor ligand gene, e.g. a preprogastrin peptide precursor gene and an EGF receptor ligand gene, e.g. a TGF α gene.

TREATMENT FOR JUVENILE DIABETES

BACKGROUND OF THE INVENTION

This invention relates to treatment of diabetes mellitus by effecting the differentiation of pancreatic islet precursor cells into mature insulin-producing cells by the combined synergistic stimulation by a gastrin/cholecystokinin (CCK) receptor ligand, particularly gastrin, and an epidermal growth factor (EGF) receptor ligand, particularly transforming growth factor alpha (TGF α).

The pancreatic islets develop from endodermal stem cells that lie in the fetal ductular pancreatic endothelium, which also contains pluripotent stem cells that develop into the exocrine pancreas. Teitelman, G. and J.K. Lee, Developmental Biology, 121: 454-466 (1987); Pictet, R. and W.J. Rutter, Development of the embryonic endocrine pancreas, in Endocrinology, Handbook of Physiology, ed. R.O. Greep and E.B. Astwood (1972), American Physiological Society: Washington D.C., p. 25-66. Islet development proceeds through discrete developmental states during fetal gestation which are punctuated by dramatic transitions. The initial period is a protodifferentiated state which is characterized by the commitment of these pluripotent stem cells to the islet cell lineage, as manifested by the expression of insulin and glucagon. These protodifferentiated cells comprise a population of committed islet precursor cells which express only low levels of islet specific gene products and lack the cytodifferentiation of mature islet cells. Pictet, R. and W.J. Rutter, supra. Around day 16 in mouse gestation, the

Diabetes, 26:632-642 (1977). Further, an abnormal persistence of pancreatic gastrin has been documented in a case of infantile nesidioblastosis. Hollande E., et al., Gastroenterology, 71:255-262 (1976). However, in neither observation was a causal relationship established between the nesidioblastosis and gastrin stimulation.

Citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

combination of factors or compositions which provide for their
in situ expression within the pancreas.

Figure 3B is a photoreproduction of the pancreatic histology of a control mouse from Example 3.

Figure 3C is a photoreproduction of the pancreatic histology of a TGF α mouse from Example 3. This field of TGF α pancreas of the study reported in Example 3 was typical and showed the interstitial cellularity and fibrosis combined with florid ductular metaplasia and has been described by Jhappan, et al. supra.

Figure 4A is a histogram graphically illustrating point-counting morphometric data which confirmed that at 17 weeks the pancreas of the INSGAS/TGF α mice had lower duct mass than the pancreas of the TGF α mice based on the study reported in Example 3.

Figure 4B is a histogram which graphically illustrates point-counting morphometric data which show that co-expression of gastrin and TGF α in the INSGAS/TGF α pancreas significantly increased the islet mass compared to the islet mass of the corresponding non-transgenic control mice. Further, TGF α expression alone does not increase islet mass. These data are based on the studies illustrated in Example 3.

islets from fetal pancreatic ducts. See Hollande, et al, Gastroenterology, 71:255-262 (1976) and Sacchi, T.B., et al., Virchows Archiv B, 48:261-276 (1985).

As used herein, the term "gastrin/CCK receptor ligand" encompasses compounds that stimulate the gastrin/CCK receptor such that when EGF receptors in the same or adjacent tissue or in the same individual are also stimulated, neogenesis of insulin-producing pancreatic islet cells is induced. Examples of such gastrin/CCK receptor ligands include various forms of gastrin such as gastrin 34 (big gastrin), gastrin 17 (little gastrin), and gastrin 8 (mini gastrin); various forms of cholecystokinin such as CCK 58, CCK 33, CCK 22, CCK 12 and CCK 8; and other gastrin/CCK receptor ligands that demonstrate the same synergistic activity with EGF receptor ligands and have a carboxy terminal peptide Trp-Met-Asp-Phe-amide which can induce differentiation of cells in mature pancreas to form insulin-secreting islet cells, when acting synergistically with an EGF receptor ligand. Also contemplated are active analogs, fragments and other modifications of the above. Such ligands also include compounds that increase the secretion of endogenous gastrins, cholecystokinins or similarly active peptides from sites of tissue storage. Examples of these are omeprazole which inhibits gastric acid secretion and soya bean trypsin inhibitor which increases CCK stimulation.

As used herein, the term "EGF receptor ligand" encompasses compounds that stimulate the EGF receptor such that when gastrin/CCK receptors in the same or adjacent tissue or in the same individual are also stimulated, neogenesis of insulin-producing pancreatic islet cells is induced. Examples of such EGF receptor ligands include EGFl-53 including EGFl-48, EGFl-52, EGFl-49 and fragments and active analogs

promoter-gastrin fusion gene construct transgenically introduced into such precursor cells. In another embodiment EGF receptor ligand stimulation is effected by expression of a EGF receptor ligand gene transgenically introduced into the mammal. Preferably, the EGF receptor ligand is TGF α and the EGF receptor ligand gene is a TGF α gene.

In another embodiment stimulation by gastrin/CCK receptor ligand and EGF receptor ligand is effected by coexpression of (i) a preprogastrin peptide precursor gene and (ii) an EGF receptor ligand gene that have been stably introduced into the mammal. Here again, the EGF receptor ligand is preferably TGF α and the EGF receptor ligand gene is preferably a TGF α gene.

In another aspect the invention relates to a method for effecting the differentiation of pancreatic islet precursor cells of a mammal by stimulating such cells with a combination of a gastrin/CCK receptor ligand, particularly gastrin, and an EGF receptor ligand, particularly TGF α . In a preferred embodiment of this aspect, gastrin stimulation is effected by expression of a preprogastrin peptide precursor gene stably introduced into the mammal. The expression is under the control of the insulin promoter. EGF receptor ligand, e.g. TGF α , stimulation is effected by expression of an EGF receptor ligand gene transgenically introduced into the mammal. In furtherance of the above, stimulation by gastrin and TGF α is preferably effected by co-expression of (i) a preprogastrin peptide precursor gene and (ii) a EGF receptor ligand, e.g. TGF α , gene that have been stably introduced into the mammal.

Another aspect of the invention is a nucleic acid fusion construct. This construct includes a nucleic acid sequence

Another aspect of this invention relates to a composition of vectors including one having the nucleic acid sequence coding for a mammalian EGF receptor ligand, e.g., TGF α , under control of a strong non-tissue specific promoter, e.g., the metallothionein promoter; and (ii) a preprogastrin peptide precursor coding sequence under control of the insulin promoter. Each vector can be a plasmid, such as plasmid pGem1 or a phage in this aspect.

Another aspect of the invention is a non-human mammal or tissue, including cells, thereof capable of expressing a stably integrated gene which encodes preprogastrin. Another embodiment of this aspect is a non-human mammal capable of coexpressing (i) a preprogastrin peptide precursor gene; and (ii) an EGF receptor ligand, e.g. TGF α , gene that have been stably integrated into the mammal, mammalian tissue or cells.

Therapeutic Administration and Compositions

Modes of administration include but are not limited to transdermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration is preferably systemic.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a therapeutic, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but

pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

Generation and Characterization of Transgenic Mice. The fragment, made as described above was prepared for microinjection as follows. It was isolated by agarose gel electrophoresis, purified by CsCl gradient purification, and dialyzed extensively against injection buffer (5mM NaCl; 0.1 mM EDTA; 5mM Tris-HCl pH 7.4). Fertilized oocytes from FVB inbred mice (Taconic Farms, Inc., supra) at the single-cell stage were microinjected using standard techniques. See Hogan, B., et al., Manipulating the mouse embryo: A laboratory manual, Cold Spring Harbor, NY (1986). Surviving embryos were then implanted into the oviducts of CD1 (Charles River Laboratories, Inc., Wilmington, MA) foster mothers according to procedures in Hogan, et al. Transgenic founder mice were identified by DNA blot techniques using DNA isolated from individual mouse tails, and a human gastrin exon 2 probe labelled with 32 dCTP by random priming. F1 mice and their siblings were similarly identified.

Homozygous MT-42 mice containing the MT-TGF α transgene derived from a CD-1 mouse strain (Jappan, supra) were crossed with heterozygotic INSGAS mice. After weaning, the offspring were placed on acidified 50mM ZnCl₂ as previously described in order to induce the metallothioneine promoter (Jhappan, supra).

Northern Blot Hybridization Assay. For Northern analysis, total RNA was extracted from tissues by the method of Cathala et al, DNA, 2:329-335 (1983). Samples of 20 μ g of total RNA were resolved on a 1% agarose denaturing gel and transferred to nitrocellulose. RNA blots were hybridized with 32 P labelled TGF α riboprobes or exon 2 of human gastrin that did not cross-hybridize with endogenous mouse gastrin mRNA.

in TGF α -induced metaplastic ductules using immunoperoxidase staining guinea pig anti-human insulin sera (Linco, Eureka, MO); a pre-immune guinea pig serum was used as a control. Immunohistochemistry was performed on 5 μ paraffin sections by the peroxidase/antiperoxidase method of Sternberger using a monoclonal rabbit antigastrin antibody. See, Sternberger, L.A., Immunocytochemistry, 2nd ed. 1979, NY: Wiley. p 104 -170.

Point-Counting Morphometrics. The relative volume of islets, ducts, or interstitial cells was quantitated using the point-counting method described in Weibel, E.R., Lab Investig., 12:131-155 (1963). At a magnification of 400 \times , starting at a random point at one corner of the section, every other field was scored using a 25 point ocular grid. An unbiased but systematic selection of fields was accomplished using the markings of the stage micrometer. Intercepts over blood vessels, fat, ducts, lymph nodes, or interlobular space were subtracted to give the total pancreatic area. A minimum of 5000 points in 108 fields (systematically chosen using the stage micrometer) were counted in each block, with the relative islet volume being the number of intercepts over islet tissue divided by the number over pancreatic tissue. The absolute islet mass or islets was calculated as the relative islet volume times pancreatic weight. See, Lee, H.C., et al, Endocrinology, 124:1571-1575 (1989).

Statistical Analysis. Differences between means were compared for significant differences using the Student's t test for unpaired data.

precursors but cannot alone effect the transition of these protodifferentiated cells into fully differentiated islets, differentiation being regulated by other factors absent from the adult pancreas.

Example 2

Pancreatic Gastrin Expression from the INSGAS Transgene

To examine the possible role of gastrin in regulating islet differentiation, transgenic mice were created that express a chimeric insulin promoter-gastrin (INSGAS) transgene in which the insulin promoter directs pancreas specific expression of the gastrin transgene (Figure 2A). Unlike the gastrin gene, insulin gene expression is not switched off after birth. Thus, the INSGAS transgene results in a persistence of gastrin expression in the adult pancreas.

The INSGAS transgene comprised 370 bp of 5' flanking DNA and the first non-coding exon of the rat insulin I gene. Cordell, B., et al., Cell, 18:533-543, 1979. It was ligated to a Bam H1-EcoR1 fragment containing 1.5 kb intron 1 and exons 2 and 3 of the human gastrin gene which encodes the preprogastin peptide precursor. Wiborg, O., et al., Proc. Natl. Acad. Sci. USA, 81:1067-1069, 1984; and Ito, et al. Proc. Natl. Acad. Sci. USA, 81:4662-4666, 1984. A 4.8 kb INSGAS fragment was isolated and microinjected into inbred FVB, one cell mouse embryos. Hogan, B. et al., Manipulating the mouse embryo: A laboratory manual, 1986, NY: Cold Spring Harbor.

Gastrin immunoreactivity in pancreatic and stomach extracts from transgenic and non-transgenic mice was assayed

n=1 1) and age matched non-transgenic controls (1.74 +/- 0.19mg, n=1 1). Thus, sustained expression of gastrin in the postnatal pancreas alone does not stimulate islet cell growth.

Example 3

Histological Examination of TGF α and TGF α /INSGAS Pancreas

Stimulation of islet growth by gastrin may require stimulation by other growth factors to create a responsive population of cells. Therefore, effects of gastrin stimulation were studied in TGF α transgenic mice which have metaplastic ducts that contain insulin expressing cells resembling protodifferentiated islet-precursors. To assess the interaction between gastrin and TGF α , three groups of mice were bred with equivalent FVB/CD1 strain genetic backgrounds: non-transgenic control, TGF α single transgenic and INSGAS/TGF α double transgenics. All three groups of mice were placed on 50mM ZnCl₂ at 3 weeks of age. At 17 weeks of age, the animals were sacrificed and the pancreas removed for histological evaluation. The pancreas from TGF α and INSGAS/TGF α mice had similar gross morphological appearances: resilient, firm and compact in contrast to the soft diffuse control pancreas. TGF α expression was equivalent in TGF α and INSGAS/TGF α groups when measured by Northern blot analysis (data not shown) and by radioimmunoassay. The pancreatic TGF α immunoreactive peptide levels were 12.2 +/- 1 and 18.9 +/- 8ng/mg protein (Mean +/- SD) in the TGF α and INSGAS/TGF α mice, respectively.

Light micrographs of hematoxylin stained paraffin sections of pancreas from the three groups of mice studied; (A: INSGAS/TGF α ; B: FVB/CD1 controls; and C: TGF α) were made. The INSGAS/TGF α pancreas had some areas of increased ductular

Expression of gastrin from the INSGAS transgene reduced the ductular metaplasia caused by TGF α over-expression. At 17 weeks, the pancreatic histology of the INSGAS/TGF α mice (Figure 3A) resembled that of the control pancreas (Figure 3B) more than that of the TGF α mice (Figure 3C).

This was confirmed by quantitating pancreatic ductular mass in the TGF α and INSGAS/TGF α transgenic mice and the FVB1/CD1 controls by point-counting morphometrics (Figure 4A). Co-expression of gastrin and TGF α in the INSGAS/TGF α pancreas also significantly increased the islet mass compared to controls (Figure 4B), whereas islet mass was not increased by expression of the TGF α or gastrin transgenes alone. The blood glucose concentration was not significantly different between the three groups of mice.

The present invention is not limited by the specific embodiments described herein. Modifications that become apparent from the foregoing description and accompanying figures fall within the scope of the claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

8. The method of claim 7 wherein such expression is expression of a preprogastrin peptide precursor gene.

9. The method of claim 1 wherein EGF receptor ligand stimulation is effected by expression of a EGF receptor ligand gene stably introduced into the individual.

10. The method of claim 1 wherein stimulation of gastrin/CCK receptor ligand and EGF receptor ligand is effected by coexpression of (i) a preprogastrin peptide precursor gene and (ii) a EGF receptor ligand gene that have been stably introduced into pancreatic cells such that they can stimulate such precursor cells.

11. A method for effecting the differentiation of pancreatic islet precursor cells of a mammal to mature insulin-secreting cells which comprises stimulating such cells with the combination of a gastrin/CCK receptor ligand and an EGF receptor ligand.

12. The method of claim 11 wherein gastrin/CCK receptor ligand stimulation is effected by expression of a preprogastrin peptide precursor gene stably introduced into such precursor cells.

13. The method of claim 12 wherein such expression is under control of an insulin expression regulatory sequence.

14. The method of claim 11 wherein EGF receptor ligand stimulation is effected by expression of an EGF receptor ligand gene stably introduced into such precursor cells.



FIG. 1B



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SUBSTITUTE SHEET (RULE 26)

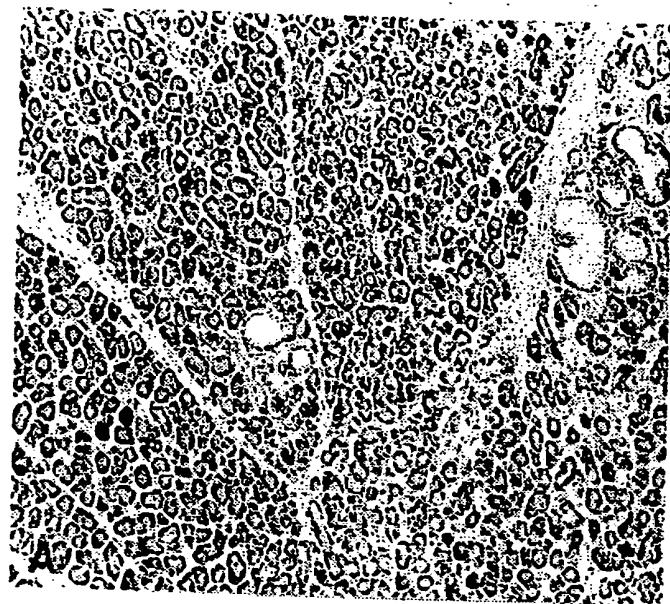


FIG. 3A

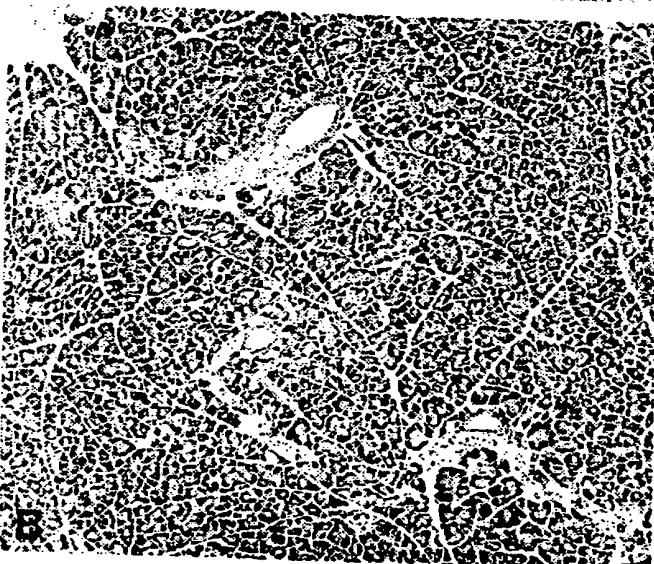


FIG. 3B

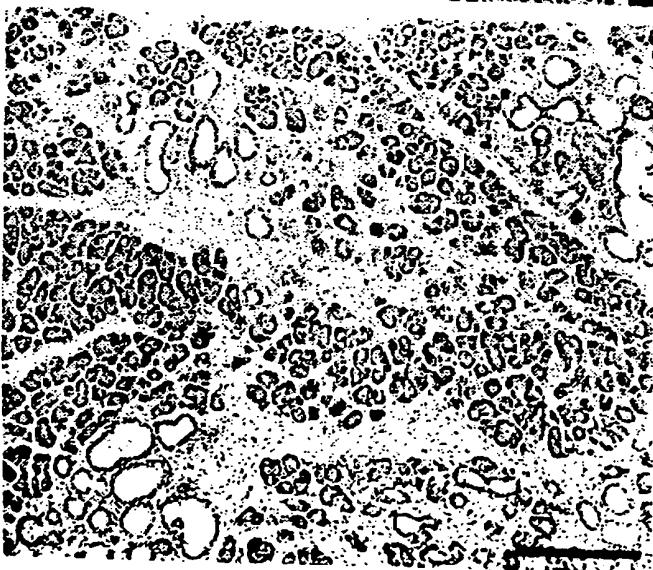


FIG. 3C

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SUBSTITUTE SHEET (RULE 26)

INTERNAL SEARCH REPORT

International application No.
PCT/US93/12055

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	J. Clin. Invest., Volume 92, issued September 1993, M. Korc, "Islet Growth Factors: Curing Diabetes and Preventing Chronic Pancreatitis?" pages 1113-1114, entire document.	1-5, 11
X,P	J. Clin. Invest., Volume 92, issued September 1993, T.C. Wang et al., "Pancreatic gastrin stimulates islet differentiation of transforming growth factor α -induced ductular precursor cells", pages 1349-1356, entire document.	1-5, 11

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-5 and 7-15 as drawn to in vivo treatment methods using EGFR and gastrin receptor ligands. This invention further includes the following distinct species which lack unity of invention:
 - A) treatment methods wherein proteins are used as the effective agent (i.e. claims 1-5, 11).
 - B) treatment methods wherein DNA is used as the effective agent (i.e. claims 1-5, 7-15).
- II. Claim 6, drawn to a treatment method using treated cells
- III. Claims 11-15, as drawn to an in vitro method of effecting cellular differentiation. This invention further includes the following distinct species which lack unity of invention:
 - A) treatment methods wherein proteins are used as the effective agent (i.e. claim 11).
 - B) treatment methods wherein DNA is used as the effective agent (i.e. claims 11-15).
- IV. Claims 16-20, drawn to DNA compositions.
- V. Claims 21 and 22, drawn to transgenic animals.

The processes of inventions I-III and likewise species of inventions I and III are separate and distinct inventions in that each is defined by a different biologically active agent and different properties and function using different method steps and results as well as having different endpoints and/or products all of which constitute the special technical features which define the contribution of each invention. Since these special technical features are not shared by each process, inventions I-III and likewise species A and B of inventions I and III do not share a technical relationship and do not form a single inventive concept within the meaning of PCT Rule 13.2.

The transgenic animals of invention V are distinct from the various processes of inventions I-III, wherein each does not require the other, and the inventions do not share a special technical relationship within the meaning of PCT Rule 13.2. Although the DNA of invention IV may be used in the processes of inventions I-III, the special technical features of those inventions are not dependent upon the DNA of invention IV, as evidenced by the claims themselves, which encompass the use of non-related proteins. Therefore, these inventions do not share a technical relationship and do not form a single inventive concept within the meaning of PCT Rule 13.2.

The products of inventions IV and V are separate and distinct. Although the DNA of invention IV may be used to make the transgenic animals of invention V, the DNA may be used in alternative processes other than the production of the transgenic animals, as evidenced by the claims and the considerations required for analysis of nucleic acid compositions are materially different than those required for transgenic animals. Therefore inventions IV and V do not share a technical relationship and do not form a single inventive concept within the meaning of PCT Rule 13.2.